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Biochemistry

A cytoplasmic 57-kDa protein that is required for translation of picornavirus RNA by internal ribosomal entry is identical to the nuclear pyrimidine tract-binding protein

(encephalomyocarditis virus/poliovirus/RNA-binding protein/translational regulation)

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ABSTRACT **Initiation of translation of the RNA genomes of picornaviruses such as poliovirus and encephalomyocarditis virus is cap-independent and results from interaction of ribosomes with a segment of the 5′ noncoding region of these mRNAs termed the internal ribosomal entry site. Genetic and biochemical studies have previously shown that a 57-kDa cytoplasmic RNA-binding protein (p57) plays an essential role in this translation mechanism. We have now found that p57 shares physical, biochemical, and antigenic properties with the pyrimidine tract-binding protein (PTB), a nuclear protein that has been implicated in various processes involving pre-mRNA. These data indicate that p57 and PTB are the same protein. Purified recombinant PTB bound specifically to a bulged hairpin within the internal ribosomal entry site of encephalomyocarditis virus and had a much lower affinity for a mutated derivative of this hairpin and for unrelated RNAs. Immunodepletion of p57/PTB from a HeLa cell-free lysate inhibited translation of poliovirus and encephalomyocarditis virus mRNAs but had no effect on translation of β-globin mRNA, confirming the essential role of p57 in translation by internal ribosomal entry.**

Translation of most eukaryotic mRNAs is initiated through interaction between the 5′-terminal methylated cap (m^7G) and the initiation factor eIF-4F, with subsequent binding of the 43S ribosomal preinitiation complex (1). Initiation of translation of the RNA genomes of picornaviruses such as poliovirus and encephalomyocarditis virus (EMCV) differs in that it is cap- and 5′ end-independent and instead results from the entry of ribosomes into an internal segment of the 5′ nontranslated region (5′ NTR; refs. 2–5). This genetic element has been termed the internal ribosomal entry site (IRES) (2, 6). Poliovirus and EMCV IRES elements are both about 450 nt long (7, 8) and highly structured (9–12) but they show little overt sequence similarity. The sensitivity of IRES function to minor structural perturbation suggests that factors that mediate internal ribosomal entry may recognize specific structural elements within the IRES (6).

IRES function is not dependent on viral gene products (2, 4), which indicates that all factors required to promote internal ribosomal entry are present in mammalian cells. It is likely that such factors could also interact with structural elements within the 5′ NTRs of some cellular mRNAs, and genetic elements functionally similar to picornavirus IRES elements have indeed been identified in other eukaryotic viral and cellular mRNAs (13–16). Internal ribosomal entry is therefore probably a eukaryotic mechanism for the initiation of translation that has been usurped by picornaviruses.

We have begun to identify trans-acting factors that interact with picornavirus IRES elements as a step in elucidating the mechanism of internal entry of eukaryotic ribosomes (7, 17). A cytoplasmic 57-kDa protein (p57) that binds to picornavirus IRES elements was thus found to be an essential trans-acting factor for their translation (7, 17–21). We report here that p57 is identical to the "pyrimidine tract-binding protein" (PTB), also known as heterogeneous nuclear ribonucleoprotein I (hnRNP I). This polypeptide has been implicated in various nuclear processes involving pre-mRNA, but no precise role for it has been established (22–31).

METHODS

Plasmid Construction. Plasmids were constructed by standard procedures (32). Plasmid pIRESΔP1 was derived by deletion of an 864-nt *Avr*II–*Avr* II fragment from pPV1(M)5′-P1 (17). The *Hin*dIII–*Nsi* I fragment of pOGβ34 (33) was inserted between the *Hin*dIII and *Pst* I sites of pBS⁻ (Stratagene) to create the plasmid pBS−(β-globin). pBS-ECAT, pBS-ECAT403M1, and pBS-ECAT403M2 have been described (3, 7). pBS-ECAT393 was derived by deletion of nt 260–392 of the EMCV 5′ NTR from pBS-ECAT. Plasmid pGEM-9Zf(−) was purchased from Promega.

Translation *in Vitro*. pBS-ECAT, pIRESΔP1, and pBS-(β-globin) were linearized at *Hpa* I, *Spe* I, and *Hin*cII sites, respectively, and were transcribed with either T7 or T3 RNA polymerase (Bethesda Research Laboratories). Synthetic mRNA transcripts were purified and their concentrations were determined (17). A cell-free extract from HeLa S3 cells was prepared for translation (4). Immunodepletion of p57 from this extract with protein A-Sepharose (Pharmacia) and PTB antiserum (5 ng/μl) that had been affinity-purified over a PTB column was monitored by UV crosslinking to an [α-^{32}P]UTP-labeled EMCV RNA probe (nt 260–488). Translation of mRNA (0.5 μg/ml) was done in 25-μl aliquots of cell-free extract with [^{35}S]methionine for 60 min at 30°C. Products of *in vitro* translation were resolved by SDS/PAGE (34) and dried gels were exposed to x-ray film.

Preparation of Cytoplasmic and Nuclear Extracts from HeLa Cells. Cytoplasmic S10 and nuclear extracts of HeLa S3 cells were prepared as described (4, 17, 35). They were adjusted to 10% (vol/vol) glycerol and stored at −80°C.

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Abbreviations: EMCV, encephalomyocarditis virus; hnRNP, heterogeneous nuclear ribonucleoprotein; IRES, internal ribosomal entry site; PTB, pyrimidine tract-binding protein; NTR, nontranslated region.

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Purification of p57 and Recombinant PTB. Recombinant human PTB-1 was overexpressed in *Escherichia coli* BL21(DE3) and was purified to apparent homogeneity by chromatography on DEAE-52 (Whatman) and poly(U)-cellulose (Pharmacia) columns (A.G. and P. Sharp, unpublished data). Murine p57 was purified to near homogeneity from the 25–40% $(NH_4)_2SO_4$ saturation fraction of the 0.2 M KCl ribosomal salt wash of Krebs-2 ascites carcinoma cells by chromatography on DEAE-52 and phosphocellulose columns (T.V.P. and E.W., unpublished data).

UV Crosslinking Assays. To generate [^{32}P]UTP-labeled RNA probes corresponding to nt 260–488, nt 393–488, nt 403–488M1, and nt 403–488M2 of the EMCV 5′ NTR, plasmids pBS-ECAT, pBS-ECAT393, pBS-ECAT403M1, and pBS-ECAT403M2 were linearized by digestion with *Hin*dIII and were transcribed with T7 polymerase (7). An RNA probe corresponding to nt 340–370 of the EMCV 5′ NTR was transcribed from synthetic DNA as described (36). A 50-nt RNA probe was transcribed with T7 polymerase from pGEM-9Zf(−) that had been linearized at the *Nsi* I site. The UV crosslinking reaction was done as described (17, 37). Immunoprecipitation of crosslinked proteins (32) was done with affinity-purified polyclonal antiserum elicited against purified recombinant PTB-1 or against a peptide corresponding to aa 219–238 of PTB-1 (26). Crosslinked polypeptides were resolved by SDS/PAGE, and dried gels were exposed to x-ray film. Relative crosslinking efficiencies were determined by scanning films with an LKB laser densitometer.

Gel slices containing purified recombinant PTB-1 or human p57 that had been crosslinked to an EMCV RNA probe (nt 393–488) and resolved by SDS/PAGE were digested with 135 mg of CNBr (Sigma) for 2 hr at 22°C or with 1 μg of *Staphylococcus aureus* V8 protease (Boehringer Mannheim) for 1 hr at 16°C, as described (38, 39).

Filter Binding Assays. Protein-excess filter binding assays were done by incubating purified recombinant PTB-1 in 25-μl reaction volumes of MMK buffer (50 mM Mes, pH 5.5/30 mM KCl/5 mM $MgCl_2$) at 25°C for 30 min. Reaction mixtures were then filtered through Schleicher & Schuell nitrocellulose filters (0.45-μm pore size) presoaked in MMK buffer, which were then washed with 200 μl of MMK buffer and dried. The retained radioactivity was counted in Econolume (NEN). Background radioactivity in the absence of protein was <5% of the input radioactivity and was subtracted in all instances. Filtration assays contained [^{32}P]UTP-labeled RNA at ≈10 pM and PTB at 5–100 nM. Equilibrium binding constants varied by less than a factor of 2 for independent replicates.

RNA Folding. Optimal and suboptimal folding for RNA sequences were computed with the "Mfold" package of programs (39–42).

RESULTS

Physical and Biochemical Properties of p57. p57 is one of several HeLa cytoplasmic proteins that were detected by UV crosslinking to ^{32}P-labeled RNA derived from the EMCV 5′ NTR. Its binding to the EMCV and poliovirus IRES elements was not dependent on ATP (unpublished observations). UV crosslinked p57 was incubated extensively with RNases and was resolved by SDS/PAGE into a doublet or triplet of bands corresponding to polypeptides of about 57 kDa (Fig. 1*A*, lanes 5 and 6) (7, 17–21). These properties of p57 and its elution profile from DEAE-cellulose, phosphocellulose, and heparin-agarose columns (T.V.P., S.H.S., and E.W., unpublished data) bear a striking similarity to characteristics of the nuclear RNA-binding protein PTB (24, 26, 28, 31). cDNAs of two rat and four human PTB isoforms have been cloned and sequenced (25, 27, 28, 31). cDNA of a 56.7-kDa murine PTB homologue, slightly smaller than the human isoforms, has also been isolated (24). The isoforms of human PTB are splice variants, and they were resolved by SDS/PAGE as a set of

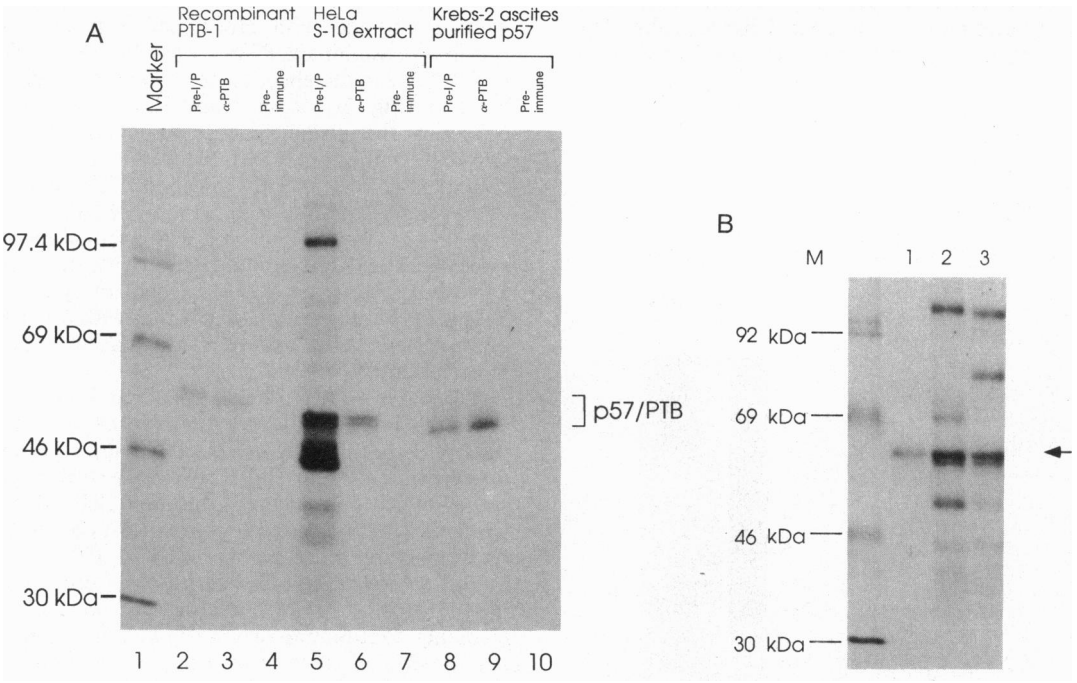

FIG. 1. Antigenic relationship between p57 and PTB. (*A*) Purified recombinant PTB (5 ng; lanes 2–4), a HeLa S10 cytoplasmic extract (50 μg; lanes 5–7), and partially purified murine p57 (lanes 8–10) were UV crosslinked to a [^{32}P]UTP-labeled EMCV 5′ NTR segment (nt 260–488). Polypeptides prior to addition of antiserum (lanes 2, 5, and 8) and those immunoprecipitated with immune (lanes 3, 6, and 9) or preimmune serum (lanes 4, 7, and 10) were resolved by electrophoresis in an SDS/10–20% polyacrylamide gradient gel. Samples used for immunoprecipitation were twice as large as those that were not treated further. The positions of ^{14}C-labeled protein standards (lane 1) are indicated at left. I/P, immunoprecipitation; α, anti. (*B*) HeLa cytoplasmic (lane 2) and nuclear (lane 3) proteins detected by UV crosslinking to a [^{32}P]UTP-labeled EMCV RNA probe (nt 393–488). Positions of p57 (lanes 2 and 3) and recombinant PTB-1 (lane 1) are indicated by the arrow.

closely migrating polypeptides after UV crosslinking to ^{32}P-labeled RNA probes derived from introns (26, 28, 30, 31). PTB is an hnRNP protein and contains four repeated domains of 80 aa that are related to ribonucleoprotein consensus-sequence RNA-binding domains (43, 44). We have explored the possibility that PTB and p57 are related proteins by using purified recombinant PTB, PTB antiserum, and EMCV IRES-specific RNA probes.

Relationship Between p57 and PTB. A p57 doublet from a HeLa S10 cytoplasmic fraction bound to an EMCV RNA probe (nt 260–488) (Fig. 1*A*, lane 5) and was precipitated with affinity-purified polyclonal antibodies (28) elicited against purified recombinant PTB (lane 6) and against a peptide corresponding to aa 219–238 of PTB-1, as well as by the monoclonal antibody 7G12 (data not shown). UV crosslinking of the same probe to recombinant PTB-1 (which contained 12 vector-derived amino acid residues and is thus larger than authentic PTB-1) yielded a single band with slightly slower mobility (lane 2) than that of p57. This single species was immunoprecipitated with PTB antiserum (lane 3), as was p57 (lane 9) that had been purified from Krebs-2 ascites carcinoma cells and crosslinked to the same EMCV probe (lane 8). Murine p57 migrated slightly faster than human p57 or PTB-1, in a manner that corresponded to its lower molecular weight. These results suggest (*i*) that a segment of the EMCV 5′ NTR can bind human and murine cytoplasmic proteins that share antigenic determinants with PTB and, conversely, (*ii*) that PTB can bind the EMCV IRES. Since PTB was identified as a predominantly nuclear protein (24–28, 31), we compared the binding of an EMCV RNA probe (nt 393–488) to HeLa cytoplasmic and HeLa nuclear extracts: comigrating 57-kDa species were detected in both cytoplasmic (Fig. 1*B*, lane 2) and nuclear (lane 3) fractions.

Additional supporting evidence for the identity of p57 and PTB was provided by the close similarity in the patterns of ^{32}P-labeled peptides resulting from cleavage of HeLa p57 and PTB by CNBr (Fig. 2*A*) and V8 protease (Fig. 2*B*). These polypeptides were digested in slices of SDS/polyacrylamide gel after UV crosslinking to a ^{32}P-labeled RNA probe. The minor differences in cleavage patterns can be attributed to the

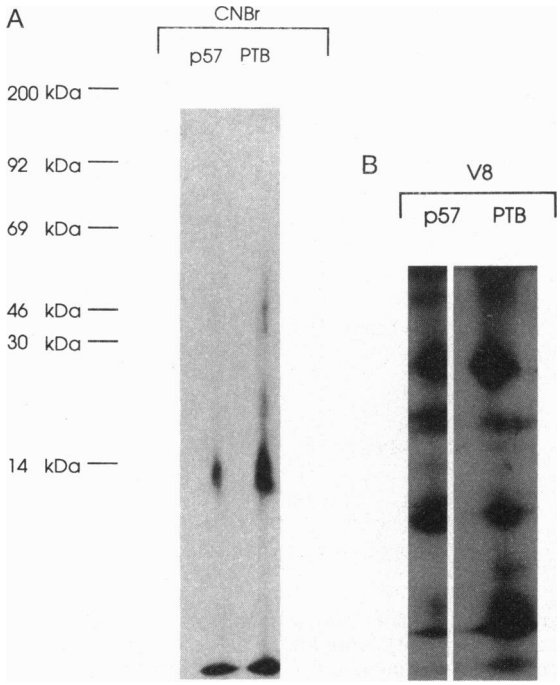

FIG. 2. CNBr (*A*) and V8 protease (*B*) cleavage patterns of PTB and p57. Cleavage products were analyzed by SDS/PAGE in 15% (*A*) and 12.5% (*B*) gels.

fact that whereas PTB-1 is a single and essentially pure recombinant polypeptide, p57 is a mixed population of polypeptides likely to consist of isoforms of PTB with similar molecular weight. The near identity of the digestion patterns eliminates the possibility that the cytoplasmic fraction of PTB merely comigrates with p57 and that a different factor of similar mobility is involved in IRES-dependent translation.

RNA-Binding Specificity of PTB. Disruption of the structure of the upper stem of domain H (nomenclature as in ref. 45) by substitution of nt 415–416 reduced UV crosslinking of p57 to an EMCV RNA probe comprising nt 403–488 (described as M1 in Fig. 3*B*). UV crosslinking was restored following additional compensatory substitution of nt 426–427 which restored the stem in the RNA probe M2 (7). Significantly, these changes correlated with the ability of mutated IRES elements in dicistronic mRNAs to direct translation (7). To analyze the binding specificity of purified recombinant PTB to IRES-specific RNAs, filter binding assays were done using the RNA probes M1 and M2, an additional EMCV RNA probe (nt 340–370) and an unrelated 50-nt transcript derived from the polylinker of pGEM9Zf(−). The latter pair of probes cannot be crosslinked to p57 in a HeLa cytoplasmic extract (G.W.W. and E.W., unpublished data). In this assay, protein–RNA complexes formed by incubating PTB and an RNA probe were detected by retention on a nitrocellulose filter. The protein concentration at half-saturation is equal to the equilibrium dissociation constant (K_d) for the reaction, if one assumes a stoichiometry of 1 and 100% active protein in RNA binding. Data for binding to RNAs M1 and M2 fit simple bimolecular binding curves with K_d = 67 nM and K_d = 20 nM, respectively. Binding to EMCV nt 340–370 and to the 50-nt polylinker transcript was below the detection limit of the assay (Fig. 3*A*). The difference in the affinity of PTB for RNA M2 over RNA M1 appeared smaller than the differences in the relative abilities of these probes to be crosslinked to rabbit p57 in reticulocyte lysate. To determine whether these differences were due to the type of assay used or to the difference in protein composition, UV crosslinking assays were performed with HeLa cell extract and pure PTB. Purified recombinant PTB-1 was able to be UV crosslinked to RNA M2 in the absence or presence of HeLa cytoplasmic proteins (Fig. 3*C*, lanes 1 and 3), an expected observation reflecting the properties of endogenous p57 (lane 2). In contrast, binding to RNA M1 was nearly undetectable for endogenous p57 and for purified PTB mixed with HeLa cytoplasmic extract (lanes 5 and 6; see also the bar graph in Fig. 3*C*), whereas it occurred with purified recombinant PTB (lane 4). The RNA-binding specificity of PTB/p57 is thus apparently increased in the presence of cytoplasmic factors. This is probably due to competition with other RNA-binding proteins, but could be due to association of p57 with (putative) cofactors.

A Role for PTB in IRES-Dependent Translation. p57 is an essential factor in translation of picornavirus mRNAs (7, 17), but not β-globin mRNA (17). If p57 and PTB were identical, immunodepletion of HeLa cell-free extracts with PTB antiserum would be expected to inhibit IRES-dependent translation of EMCV and poliovirus mRNAs but to have no effect on translation of β-globin mRNA. PTB-1 antiserum did indeed inhibit translation of mRNAs containing poliovirus (Fig. 4, lanes 2 and 3) and EMCV (lanes 17 and 18) IRES elements. Preimmune serum had no effect (lanes 6, 7, 19, and 20). A similar albeit less pronounced inhibition of IRES-dependent translation occurred with the antiserum elicited against a peptide corresponding to aa 219–238 of PTB-1 (lanes 4 and 5). Translation of uncapped and capped β-globin mRNA was unaffected by addition of PTB-1 antiserum (lanes 10 and 11), the peptide antiserum described above (lanes 8 and 9), or preimmune serum (lane 13). This result was confirmed by cotranslating poliovirus IRES-dependent

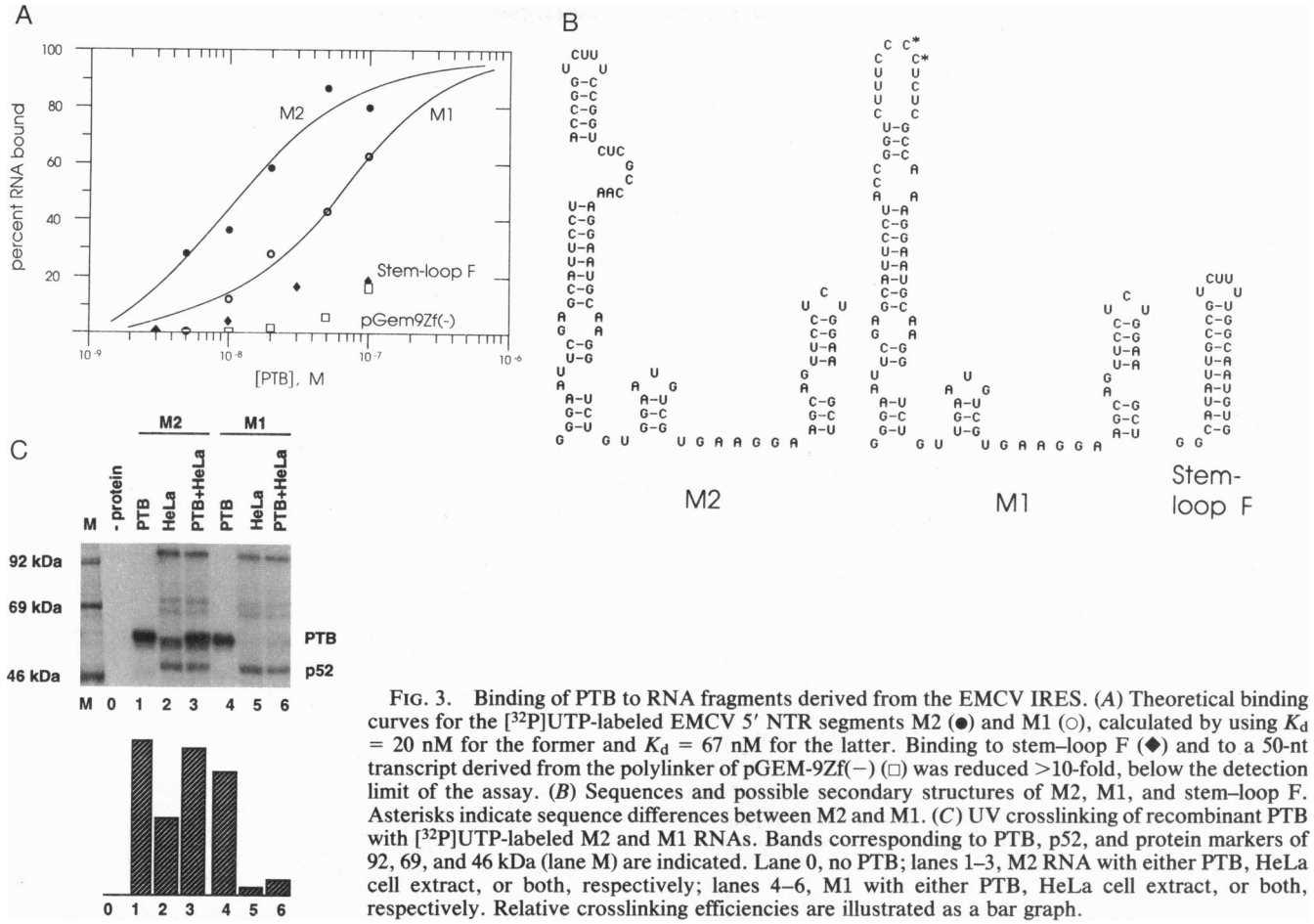

FIG. 3. Binding of PTB to RNA fragments derived from the EMCV IRES. (*A*) Theoretical binding curves for the [^{32}P]UTP-labeled EMCV 5′ NTR segments M2 (●) and M1 (○), calculated by using K_d = 20 nM for the former and K_d = 67 nM for the latter. Binding to stem–loop F (◆) and to a 50-nt transcript derived from the polylinker of pGEM-9Zf(−) (□) was reduced >10-fold, below the detection limit of the assay. (*B*) Sequences and possible secondary structures of M2, M1, and stem–loop F. Asterisks indicate sequence differences between M2 and M1. (*C*) UV crosslinking of recombinant PTB with [^{32}P]UTP-labeled M2 and M1 RNAs. Bands corresponding to PTB, p52, and protein markers of 92, 69, and 46 kDa (lane M) are indicated. Lane 0, no PTB; lanes 1–3, M2 RNA with either PTB, HeLa cell extract, or both, respectively; lanes 4–6, M1 with either PTB, HeLa cell extract, or both, respectively. Relative crosslinking efficiencies are illustrated as a bar graph.

mRNA (ΔP1) and β-globin mRNA in a HeLa cell-free extract, using either preimmune serum or the monoclonal antibody 7G12 (specific for PTB/hnRNP I; ref. 27). Whereas translation of ΔP1 was progressively inhibited by increasing amounts of this monoclonal antibody, that of β-globin was unaffected (unpublished observations). However, IRES-dependent translation was not restored by addition of purified PTB to HeLa extracts that had been immunodepleted with PTB antiserum (unpublished observations). The inability to restore IRES function after immunodepletion probably reflects the disruption or elimination of a ribonucleoprotein complex that cannot be reversed by addition of PTB alone. Characterization of the role of p57/PTB in internal ribosomal entry in a translation assay system may thus require supplementation with additional factors to reconstitute activity.

DISCUSSION

The results presented above demonstrate that p57 and PTB have common physical, biochemical, and antigenic properties, thus leading to the conclusion that they are the same protein. Initiation of picornavirus translation occurs in the cytoplasm, and the involvement of PTB in this process is unexpected, since previous reports have implicated it in various nuclear events (22–31). However, it is consistent with the detection of PTB in the cytoplasm of HeLa cells by immunofluorescence (27), an observation that we have confirmed (M.S. and E.W., unpublished data).

It is not unusual that viruses usurp cellular proteins to serve virus-specific functions that differ from their normal cellular functions (46, 47). However, it is possible that PTB may also be involved in the translation of selected cellular mRNAs, perhaps of a type such as BiP mRNA, which can function in a cap-independent manner (16), and that PTB can therefore be considered a bona fide translation initiation factor. An implicit requirement of translation initiation by internal ribosomal entry is recognition of internal elements within the 5′ NTR of an mRNA by either RNA or protein

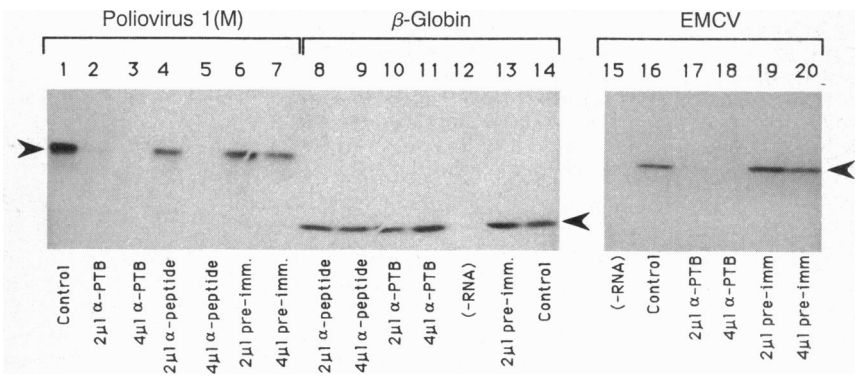

FIG. 4. Inhibition of IRES-dependent translation by PTB antiserum. Translation of uncapped poliovirus (pIRES-ΔP1) (lanes 1–7), β-globin (lanes 8–14), and EMCV (EMCV IRES element with chloramphenicol acetyltransferase coding sequence, pBS-ECAT) (lanes 15–20) mRNAs was assayed in lysate treated with 2 μl (lanes 2, 10, and 17) or 4 μl (lanes 3, 11, and 18) of PTB antiserum, 2 μl (lanes 4 and 8) or 4 μl (lanes 5 and 9) PTB-specific antipeptide serum, 2 μl (lanes 6, 13, and 19) or 4 μl (lanes 7 and 20) of preimmune serum, or without serum addition (lanes 1, 14, and 16). Lanes 12 and 15, lysate incubated without exogenous mRNA. Poliovirus ΔP1, β-globin, and ECAT translation products (arrowheads) were resolved by SDS/10–20% PAGE.

factors. Nuclear gene-encoded polypeptides have been identified that promote the translation of specific mRNAs in mitochondria and chloroplasts, but factors that bind selectively to cytoplasmic mRNAs and function to promote initiation of translation were not identified (48). p57/PTB is a likely candidate to promote interaction between ribosomes and such internal RNA sequences, because it is associated with ribosomes (7, 17) and binds a specific structural domain (nt 404–488) within the EMCV IRES with high specificity (ref. 7; Fig. 3). PTB contains four repeated domains, each consisting of 80 aa, that can be aligned with ribonucleoprotein consensus-sequence RNA-binding domains (27, 44), and could therefore make additional contacts with other sequences within an mRNA. Multiple noncontiguous p57-binding domains have been identified within picornavirus IRES elements (refs. 7 and 19–21; unpublished observations), but the stoichiometry of RNA binding by PTB has not been characterized.

Several translation factors, including the eukaryotic initiation factors eIF-4F (49) and eIF-4E and eIF-2 (50), have been detected in the nucleus, where they may participate in processes such as nucleocytoplasmic transport. The involvement of PTB in both nuclear and cytoplasmic aspects of RNA metabolism is therefore not without precedent. A precise role for PTB has not been established, but it is associated with specific cofactors and is involved in the assembly of ribonucleoprotein complexes that bind pre-mRNAs (22–24, 31). Cytoplasmic p57/PTB could play an analogous role with cofactors such as p52 (37), eIF-2 (51, 52), or "initiation correction factor" (53) in the formation of an IRES-related ribonucleoprotein complex, or "IRESome," that promotes interaction of the 40S ribosomal subunit with RNA sequences within the 5′ NTR.

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